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Analysis of the Reaction Steps in the Bioconversion of D,L-ATC to L-Cysteine

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The reaction steps involved in the bioconversion of a chemically synthesized precursor, D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid (D,L-ATC), to L-cysteine and the properties of the involved enzymes were investigated. It was found that the conversion consisted of two steps, *i. e.*, D,L-ATC to S-carbamyl-L-cysteine (S-C-L-cysteine) and S-C-L-cysteine to L-cysteine, and the S-C-L-cysteine was an intermediate between them. While the enzymes involved in the reactions were induced by the addition of D,L-ATC as an inducer, S-C-L-cysteine induced only the enzyme involved in the latter step. The conversion of S-C-L-cysteine to L-cysteine could be also carried out in the presence of hydroxylamine and its rate was much faster than that by the corresponding enzyme. On the other hand, L-cysteine (or L-cystine) was decomposed to evolve H_2S by the enzyme considered to be a kind of desulfhydrase. However, hydroxylamine was a perfect inhibitor for this enzyme.

L-Cysteine, a sulfur-containing amino acid, has been used in medicines, cosmetics, and food additives, *etc.* Traditionally, it has been produced mainly *via* acid-or alkali-hydrolysis of hairs. However, this method has several disadvantages such as low yields and unpleasant odors (2). On the other hand, chemical synthesis of L-cysteine requires many complicated steps including optical resolution (4).

Recently, there have been some reports about a combined synthesis using both chemical and enzymatic methods (5-8). This paper deals with the bioconversion of a chemically synthesized precursor, D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid, to L-cysteine. Specifically, the reaction steps and the properties of the enzymes involved in the bioconversion were extensively investigated.

MATERIALS AND METHODS

Chemicals

D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid (D,L-ATC) was obtained from Korea Research Institute of Chemical Technology. S-Carbamyl-L-cysteine (S-C-L-cysteine), L-cysteine and L-cystine were the products of Sigma. Cellulose plate, silicagel plate and ninhydrin spray reagent were purchased from Merck.

Strain and Cultivation

For a seed culture, *Pseudomonas* sp. CU6 (5) was inoculated into a 250 m*l* Erlenmeyer flask containing 25 m*l* medium (Table 1) and incubated at 30℃ for overnight. For the main culture, 4 m*l* of the seed broth was transferred to 50 m*l* medium in a 500 m*l* Erlenmeyer flask and cultivated at 30℃ on a rotary shaker. After 4 hours of cultivation, 0.15% D,L-ATC was added in order to induce the relevant enzymes in the cells, and then the cells were further cultivated for 5 hours at 28℃.

Preparation of Crude Enzyme

The cells harvested at the end of cultivation were cen-

Table 1. Composition of medium

Components	Contents (w/v, %)
Glucose	2.0
Yeast extract	0.5
Peptone	0.5
NaCl	0.25
KH_2PO_4	1.0
$MgSO_4$	0.05
$FeSO_4$ (pH 7.0 with NaOH)	0.0007

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trifuged, washed with 0.1 M phosphate buffer solution (pH 7.8) and sonicated in the same buffer solution. The filtrated solutions were used as the enzyme source in these experiments.

Reaction Procedures

As a reaction mixture, one milliliter of crude enzyme solution was added to 1.5 m*l* of D,L-ATC solution and it was incubated at 33℃. The reaction was stopped by adding 0.5 m*l* of 6 N hydrochloric acid. The D,L-ATC solution was prepared by mixing 1 m*l* of the solution containing 1.5% D,L-ATC and 1.0% KH_2PO_4 with 0.5 m*l* of 150 mM hydroxylamine solution. For the reactions carried out without hydroxylamine, it was substituted by 0.1 M phosphate buffer solution.

For the reaction using S-C-L-cystene as a substrate, S-C-L-cysteine was prepared in the same manner as D,L-ATC solution and used in place of D,L-ATC. On the other hand, the degradation reaction of L-cysteine was carried out in a solution having the ratio of 2:2:1 for 8 mM L-cysteine solution, enzyme solution and 0.1 M phosphate buffer solution.

Measurements of Substrate and Product Contents

D,L-ATC: D,L-ATC content was determined by thin layer chromatography(TLC). Samples were developed on silicagel plate using a solvent mixture (butanol : water : acetic acid=4:1:1) and the compound was detected at 235 nm by TLC scanner (Shimadzu CS-9000).

L-Cysteine: TLC method consisting of cellulose plate and a solvent mixture (ethanol : water:acetic acid: 28% aminoia water : trichloroacetic acid=55 m*l* : 35 m*l* : 2 m*l* : 3.5 g) was used with 0.1% ninhydrin spray reagent for the determination of L-cysteine. The absorbances were measured at 560 nm by TLC scanner. Alternatively, Gaitonde's chemical method was used (1).

Sulfide: The method employed by Kredich *et al.* (3) was used. After adding 0.2 m*l* of 7.2 N hydrochloric acid solution containing 0.02 M N,N'-dimethylphenylenediamine sulfate to 2 m*l* of the reacted solution, 2 m*l* of 0.03 M ferric chloride solution was added and the absorbance was measured at 650 nm by spectrophotometer.

RESULTS AND DISCUSSION

Since it was expected that the conversion of D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid (D,L-ATC) to L-cysteine would be carried out *via* two consecutive enzyme reaction steps, efforts were made to identify if an intermediate existed between the substances. After the reaction of D,L-ATC as a substrate with the crude enzyme solution was carried out for 4 hours, the products formed were compared with the standard samples of L-cysteine and S-carbamyl-L-cysteine (S-C-L-cysteine) on thin layer chromatograms. As shown in the product chromatograms (B) of Fig. 1, together with the spots of L-cystine, another one appeared which was considered to be an intermediate between D,L-ATC and L-cysteine. And it was supposed to be S-C-L-cysteine because it showed the same R_f value as that of a standard S-C-L-cysteine.

In the next, S-C-L-cysteine was used as a substrate for L-cysteine-forming reaction in order to verify whether L-cysteine was produced from it. From the fact that the chromatograms D′ and D″ in Fig. 2 showed the spots of L-cysteine and L-cystine, it became clear that S-C-L-cysteine was converted to L-cysteine (or L-cystine). It strongly suggests that S-C-L-cysteine would be the intermediate.

On the other hand, the conversion of S-C-L-cysteine to L-cysteine could be carried out in another way, *i.e.*, *via* the reaction in the presence of hydroxylamine instead of the corresponding enzyme. The chromatograms C′ and C″ show that the reaction with hydroxylamine was much more effective than that by the enzyme (D′ and D″). Besides, the L-cysteine could be formed spontaneously, but at a pretty low rate, as shown in the chromatograms A′ and A″.

The L-cysteine formed thus was easily oxidized to L-cystine under aeration. The fact that the cysteine was produced only in L-form could be verified from the com-

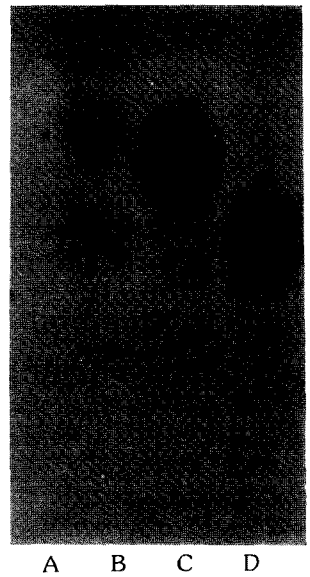

Fig. 1. Thin layer chromatograms of S-carbamyl-L-cysteine, L-cystine and enzyme-reacted samples.
A; Enzyme reaction was not performed.
B; Enzyme reaction was performed for 4 hrs.
C; Standard S-carbamyl-L-cysteine.
D; Standard L-cystine.

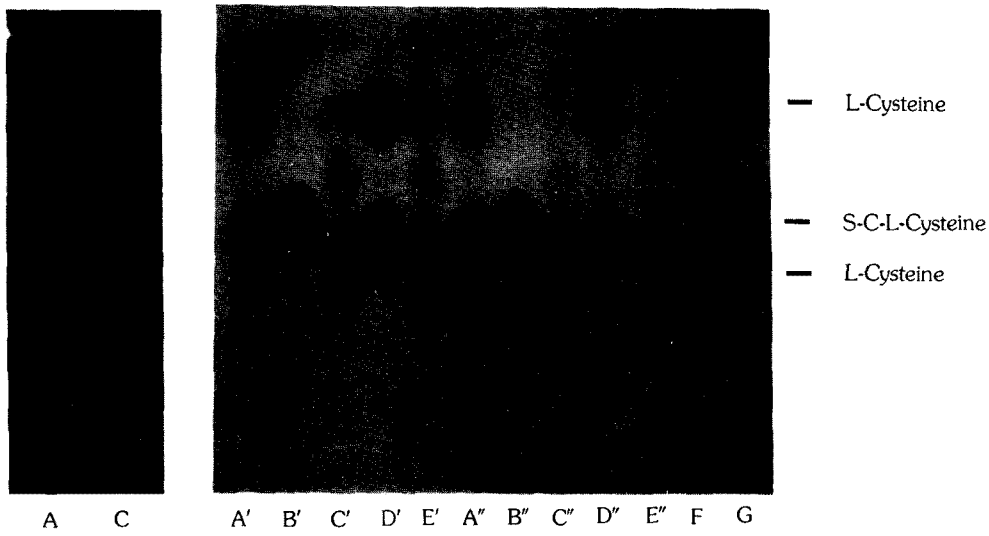

Fig. 2. Thin layer chromatograms of the products from the reaction using S-C-L-cysteine as a substrate.
A, A′ and A″; S-Carbamyl-L-cysteine (S-C-L-Cysteine)
B and B″; S-C-L-cysteine+HCl
C, C′ and C″; S-C-L-cysteine+Hydroxylamine
D′ and D″; S-C-L-cysteine+Enzyme
E′ and E″; S-C-L-cysteine+Hydroxylamine+Enzyme
F; L-Cysteine
G; L-Cystine
*A and C; No reaction (Control).
A′, B′, C′, D′ and E′; After 1 hr reaction.
A″,B″,C″,D″ and E″; After 6 hrs reaction.

parisons in polarity between standard L-cystine and the samples (Table 2), because they were similar in specific optical rotation degree. Little differences between them were considered to come from the impurities of the samples in which cellular materials existed.

It was previously reported by our group that the L-cysteine (or L-cystine) formed had been degraded by a decomposing enzyme in the crude enzyme solution and hydroxylamine functioned as an inhibitor of the enzyme (5). Fig. 3 shows that the addition of hydroxylamine could completely prevent the L-cysteine from being degraded. In the absence of that, however, most of L-cysteine was decomposed in an hour to evolve pyruvate (data were not shown) and H_2S, and for this reason, the enzyme was considered to be a kind of desulfhy-

Table 2. Comparison of the polarities of standard L-cystine and samples

Concentration (w/v, %)	$[\alpha]_D^{24°}$	
	Standard L-cystine	Sample
0.05	−219.78	−208.22
0.10	−219.06	−199.13
0.15	−216.00	−198.42

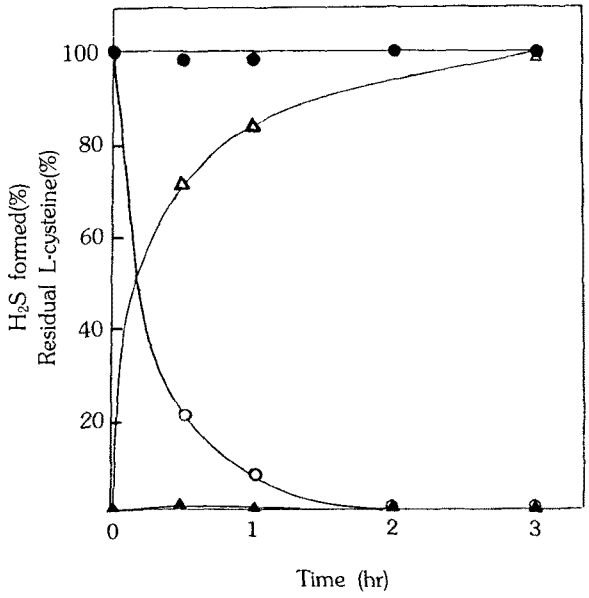

Fig. 3. Decomposition profile of L-cysteine.
○; Residual L-cysteine without hydroxylamine.
●; Residual L-cysteine with hydroxylamine.
△; H_2S without hydroxylamine.
▲; H_2S with hydroxylamine.

Tab le 3. Induction of the enzymes involved in the conversion of D,L-ATC to L-cysteine

Substrate	Inducer		
	ATC	S-Carbamyl-L-cysteine	None
ATC	+*	−	−
S-Carbamyl-L-cysteine	+	+	−

*+: L-Cysteine was produced.
−: L-Cysteine was not produced.

drase, *i.e.*, L-cysteine desulfhydrase.

Finally, it was further investigated whether the two enzymes involved in the conversion of D,L-ATC to L-cysteine were inducibly or constitutively formed in cells. Inducers such as D,L-ATC or S-C-L-cysteine were fed to the medium during cell growth and inductions of each enzyme were checked. As shown in Table 3, while D,L-ATC induced both enzymes, S-C-L-cysteine induced only the enzyme of the latter step. These results indicate that both enzymes are inducible and these two enzymes are regulated in a separate way. And the fact that S-C-L-cysteine could induce only the latter enzyme supports again that it is an intermediate between D,L-ATC and L-cysteine.

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